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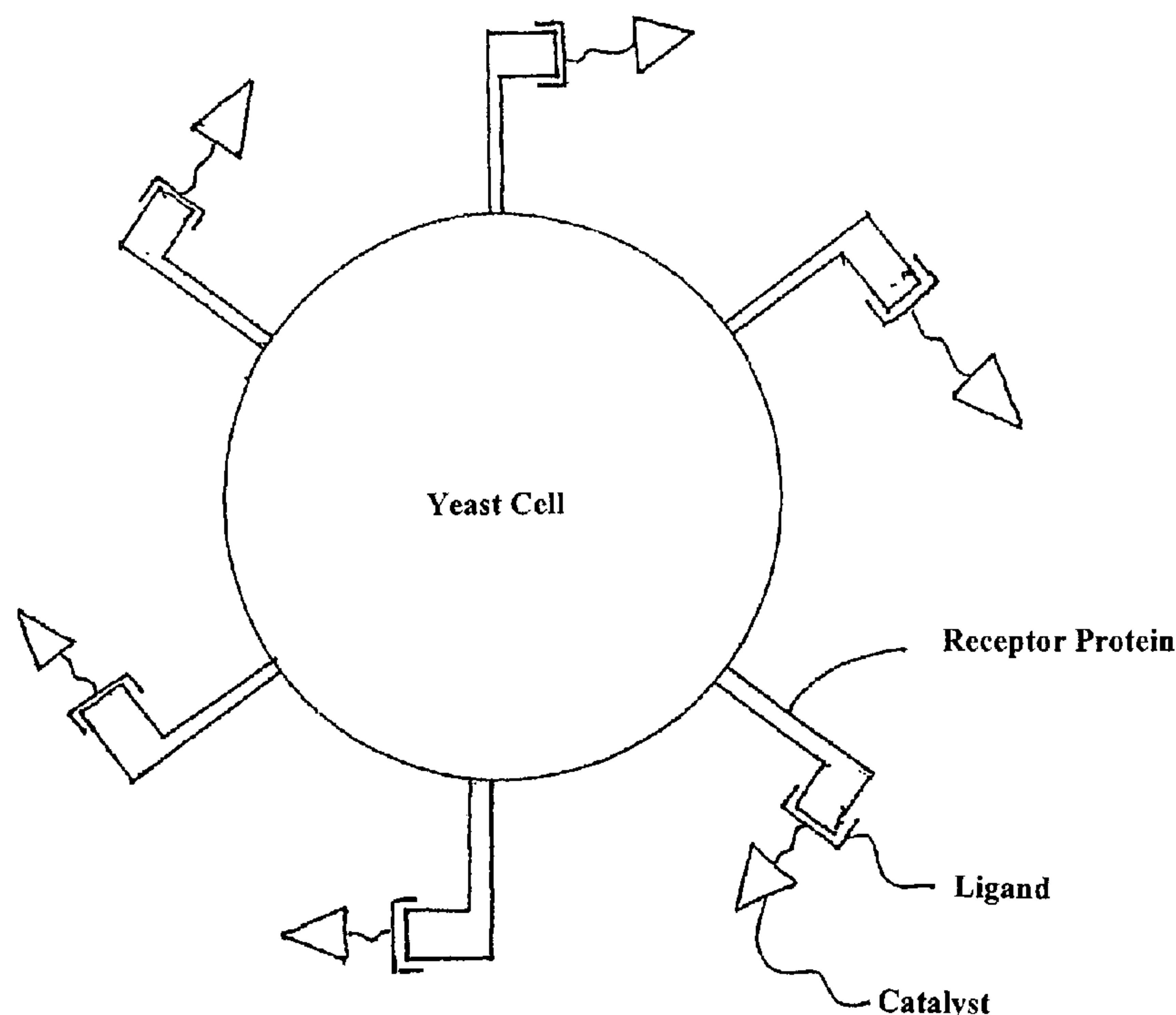
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(54) Titre : UTILISATION DE PROTEINES DE PRESENTATION DE SURFACE POUR PRESENTER DES
CATALYSEURS INORGANIQUES SUR LA SURFACE D'UNE LEVURE, D'UNE BACTERIE OU D'UN VIRION

(54) Title: USE OF SURFACE DISPLAY PROTEINS TO DISPLAY INORGANIC CATALYSTS ON THE SURFACE OF A
YEAST, BACTERIUM OR VIRION



(57) Abrégé/Abstract:

Methods and compositions for providing inorganic catalysts displayed on the surface of biological particles such as a cell or virion are described. In an exemplary embodiment the biological particle is a yeast cell including one or more receptor proteins uniformly displayed on the surface of the yeast *Saccharomyces cerevisiae*. Each receptor protein is anchored to a ligand that is selective for that protein, and each ligand is, in turn, bound to a catalyst. Other contemplated embodiments include where the biological particles are other yeasts, bacterial cells or virions where a protein may be displayed on the surface and linked to a ligand that binds the inorganic catalyst.

ABSTRACT OF THE DISCLOSURE

Methods and compositions for providing inorganic catalysts displayed on the surface of biological particles such as a cell or virion are described. In an exemplary embodiment the biological particle is a yeast cell including one or more receptor proteins uniformly displayed on the surface of the yeast *Saccharomyces cerevisiae*. Each receptor protein is anchored to a ligand that is selective for that protein, and each ligand is, in turn, bound to a catalyst. Other contemplated embodiments include where the biological particles are other yeasts, bacterial cells or virions where a protein may be displayed on the surface and linked to a ligand that binds the inorganic catalyst.

USE OF SURFACE DISPLAY PROTEINS TO DISPLAY INORGANIC CATALYSTS ON THE SURFACE OF A YEAST, BACTERIUM OR VIRION

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0001] The present invention relates to the use of surface display technology as a uniform catalyst support for synthetic catalysts. Catalyst supports of the invention include uniform layers of catalyst on their surface, allowing catalysis with properties of homogeneous catalysts, while still allowing efficient separation and recycling of the catalyst. Although a variety of biological supports are taught herein, yeast cell-supported catalysts are particularly preferred.

2. Background

A. Cell Surface Displays

[0002] A number of microorganisms and virions may be engineered so that proteins are genetically displayed on their surface. The yeast *Saccharomyces cerevisiae* is often used for this process. A number of methods, structures, and applications of yeast cell-surface display have been reported. Kondo, A., and Ueda, M., "Yeast Cell-Surface Display - Applications of Molecular Display," *Appl. Microbiol. Biotechnol.* 64:28-40 (2004); Ueda, M. & Tanaka, A., "Cell Surface Engineering of Yeast: Construction of Arming Yeast with Biocatalyst" *J. Biosci. and Bioeng.* 90(2): 125-136 (2000).

[0003] Boder and Wittrup report that *S. cerevisiae* is ideal for cell surface display of proteins, because *S. cerevisiae* possesses both secretory machinery and protein folding mechanisms that are similar to those of mammalian cells. (Boder, E.T. & Wittrup, D.K., "Yeast Surface Display for Screening Combinatorial Polypeptide Libraries," *Nature Biotech.* 15:553-557 (1997)). Other benefits include large numbers of surface fusions per

cell and the ability to test the cells using flow cytometry. (Boder, *et al.*). Yeast cells may display proteins of differing stability and expression level, as reported by Park, *et al.*, "Limitations of Yeast Surface Display in Engineering Proteins of High Thermostability," *PEDS*, 19(5): 211-217 (2006).

[0004] Yeast cell surface display technology is further reported in U.S. Patent No. 6,423,538, to Wittrup, *et al.*, "Yeast Cell Surface Display of Proteins and Uses Thereof." Wittrup, *et al.*, reports a genetic method for anchoring polypeptides to a yeast cell wall. U.S. Patent No. 6,300,065, to Kieke, *et al.*, "Yeast Cell Surface Display of Proteins and Uses Thereof," reports a method for the fusion of the N-terminus of a "polypeptide of interest" to the C-terminus of the yeast Aga2p cell wall protein.

[0005] Kieke's method may purportedly be used for anchoring an scFv antibody fragment to exterior of the yeast cell wall. Yeast cell surface display has also been reported for use in antibody epitope mapping. Chao, *et al.*, "Fine Epitope Mapping of Anti-Epidermal Growth Factor Receptor Antibodies Through Random Mutagenesis and Yeast Surface Display," *J. Mol. Biol.*, 342(2): 539-550 (2004); *see also* Colby, *et al.*, "Development of a Human Light Chain Variable Domain (V_L) Intracellular Antibody Specific for the Amino Terminus of Huntingtin *via* Yeast Surface Display," *J. Molec. Biol.* 342(3): 901-912 (2004); Min Li, "Applications of Display Technology in Protein Analysis," *Nature Biotech.* 18:1251-1256 (2000); Feldhaus, M.J. & Siegel, R.W., "Yeast Display of Antibody Fragments: A Discovery and Characterization Platform," *J. Immunol. Methods* 290(1-2): 69-80 (2004); Weaver-Feldhaus, *et al.*, "Directed Evolution for the Development of Conformation-Specific Affinity Reagents using Yeast Display" 18(11):

527-536 (2005); Little, *et al.*, "Bacterial Surface Presentation of Proteins and Peptides: An Alternative to Phage Technology?" *Trends in Biotechnology* 11 (1993).

[0006] Cell surface display has also reportedly been used for absorption of environmental metals. Wernerus, H. & Stahl, S., "Biotechnological Applications for Surface-Engineered Bacteria" *Biotechnol. Appl. Biochem.* 40:209-228 (2004). Cell surface display has also reportedly been used to bind eukaryotic cell display libraries to solid surfaces. Peelle, *et al.*, U.S. Pat. Appl'n No. US2006/0003387, "Cell Display Libraries"; Andres, *et al.*, "Immobilization of *Saccharomyces cerevisiae* Cells to Protein G-Sepharose by Cell Wall Engineering" *J. Mol. Microbiol. & Biotech.* 5(3) 161-166 (2003). Expression of the ZZ domain from *Staphylococcus aureus*, which binds to the Fc part of immunoglobulin G (IgG) has also been reported. Shimojyo, *et al.*, "Preparation of Yeast Strains Displaying IgG Binding Domain ZZ and Enhanced green Fluorescent Protein for Novel Antigen Detection Systems" *J. Biosci & Bioeng.* 96(5):493-495 (2003).

[0007] Kondo reports that yeast cell surface display has been used for biocatalysis; however, Kondo's biocatalysts have a number of disadvantages that hinder their general applicability. For example, the biocatalysts are bound directly to the surface of the yeast cell. Such catalysts may not be interchanged for other catalysts as the need arises, and familial yeast lines are limited to a single biocatalyst configuration. Furthermore, biocatalyst that is adsorbed to an unwanted target is difficult or impossible to remove or separate from a cell without irrevocably damaging the cell. Biocatalysts are also traditionally limited to those catalysts that may be expressed directly on the cell surface, such as protein molecules or enzymes.

[0008] Cell surface display using microorganisms other than yeast has also been reported. For example, Wang, *et al.*, "Specific Adhesion to Cellulose and Hydrolysis of Organophosphate Nerve Agents by a Genetically Engineered *Escherichia coli* Strain with a Surface-Expressed Cellulose-Binding Domain and Organophosphorus Hydrolase," *App. and Env. Microbiol.* 68(4): 1684-1689 (2002), reports degradation of parathion and paraoxon using a strain of *E. coli* displaying organophosphorus hydrolase (OPH) and a cellulose-binding domain. The OPH was expressed on the cell surface using the Lpp-OmpA fusion system or the truncated ice nucleation protein anchor.

B. Catalyst Supports

[0009] There is a need for catalyst supports that are uniform in size and that have the catalyst distributed evenly over the surface of the catalyst support. Uniform supports offer a number of advantages, including minimizing the effects of diffusion and differences between catalyst sites. Catalyst supports may also aid in convenient handling of some catalysts.

[0010] One example of generally uniform inorganic catalyst supports may be found in United States Patent Application Publication No. 2006/0009354, to Yueng, *et al.* The '354 publication reports a catalytic material comprising a metal catalyst anchored to a metal oxide crystal. The metal catalyst is anchored to the surface of the metal oxide by interacting with a hydroxyl group on the surface of the metal oxide.

[0011] Catalysts such as those reported in the '354 publication have a number of disadvantages. For example, they may be unsuitable for organic use. Furthermore, they may allow only the use of metal catalysts. Their production may also be expensive, and

their construction may make catalyst regeneration difficult. Each support may be limited to a single catalyst.

[0012] Embodiments of the invention provide catalysts and catalyst supports that may address one or more of the above disadvantages of the prior art. Methods of construction, use, and regeneration of those catalysts are also provided.

SUMMARY OF THE INVENTION

[0013] The present invention provides a catalytic material comprising one or more receptor proteins disposed from the surface of a biological support. In a typical embodiment, the receptor proteins are disposed uniformly over the surface of the biological support. The receptor proteins may be identical proteins, or they may be different proteins. Each receptor protein is linked to an anchoring protein that is engaged with surface of the biological support.

[0014] In a typical practice, the receptor protein is linked to the anchoring protein in the form of a fusion protein, whereby amino acids of the anchoring protein domain are linked as a continuous polypeptide sequence with amino acids of the receptor protein, thereby forming a heterologous fusion protein having an anchoring domain from the anchoring protein and ligand binding domain from the receptor protein. The anchoring domain may engage the surface of the biological support by binding to, being adsorbed within, or being cross-linked to, other proteins or carbohydrates disposed on the surface of the biological support. Typically, when the biological support is a cell, the anchoring domain is embedded in the outer membrane of the cell or binds to a cell wall protein or carbohydrate. Depending on the anchoring protein used, the anchoring domain may be

partly disposed within the surface of the biological support, for example in the form of a peripheral membrane protein, or may extend through the biological support surface, for example, in the form of an integral membrane protein. Each receptor protein contains a ligand binding domain that is further bound to a ligand, and each ligand in turn is bound to a catalyst. Receptor-ligand binding and ligand-catalyst binding may be accomplished by covalent (e.g. chemical cross-linking) or non-covalent binding

[0015] The invention further provides a catalyst support system comprising one or more receptor proteins displayed on the surface of a biological support. The receptor proteins are thereby typically displayed uniformly over the surface. The receptor proteins may be identical proteins or different proteins. The receptor proteins are selected to bind to a selected ligand, which in turn is selected to bind to a selected catalyst to be supported.

[0016] The invention also provides a method for making a catalytic material and/or a catalyst support system as described above. One or more receptor proteins are expressed as fusion proteins with anchoring proteins and distributed about the surface of a biological support. In one embodiment the proteins are identical proteins, and in another embodiment they are one or more different proteins. In one embodiment the proteins are displayed uniformly about the surface of a biological support, forming a catalyst support material. One or more catalyst molecules are bound to respective ligands to form ligand/catalyst constructs. The ligands are designed or chosen to selectively bind or be cross-linked to the binding domain of the receptor proteins, referred to herein as "tethering". The ligand/catalyst constructs and the biological supports displaying the receptor protein(s) are mixed, allowing the ligand/catalyst constructs to be tethered to the receptor proteins, forming a catalytic material.

[0017] Biological supports may be selected from, for example, virions and microorganisms. Microorganisms may be, for example, bacteria or yeasts. Bacteria may be, for example, from the genus *Escherichia*, *Corynebacterium*, *Bacillus*, or *Lactococcus*. *Escherichia coli* is a preferred bacterium. Yeasts for use in the invention may include those, for example, from the genus *Saccharomyces*, *Pichia* or *Candida*. A preferred yeast is *Saccharomyces cerevisiae*.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0018] **Figure 1** schematically depicts a two-dimensional view of a single yeast cell surface display catalyst system of the invention.

[0019] **Figure 2** shows yeast cell surface display systems using **A** α -agglutinin (SEQ ID NO: 1), **B** a-agglutinin (SEQ ID NO: 2), **C** C-terminus region of Flo1p (SEQ ID NO: 3), and **D** N-terminus flocculation function domain of Flo1p (SEQ ID NO: 3). (Kondo, *supra.*; and van der Vaart, J. M., *et al.*, "Comparison of Cell Wall Proteins of *Saccharomyces cerevisiae* as anchors for Cell Surface Expression of Heterologous Proteins," *App. & Env. Microbiol.* 63(2): 615-620 (1997)).

[0020] **Figure 3** shows a catalytic support material comprising a yeast cell and one or more of receptor proteins exhibited on the surface of the yeast cell.

[0021] **Figure 4** shows plasmid pCT302 (Boder & Wittrup), which may be used to express a receptor protein as a fusion to the yeast Aga2p agglutinin mating protein under the control of the GAL1,10 galactose-inducible promoter. The N-terminal flanking sequence of pCT302 is shown as SEQ ID NO: 4, and the C-terminal flanking sequence is shown as SEQ ID NO: 5.

[0022] Figure 5 shows a map of plasmid pYD1 (SEQ ID NO: 6), which is 5009 nucleotides available from InVitrogen Life Technologies (Grand Island NY). The enumerated positions for the relevant elements are as follows: GAL1 promoter (1-451); T7 promoter (475-494); AGA2 ORF (534-794); pYD1 forward priming site (751-770); Gly/Ser linker (507-857); Xpress™ epitope (891-914); multiple cloning site ((914-992); V5 epitope (993-1034); polyshistidine region (1044-1061); pYD1 reverse primer site (1114-1098); Mat transcription termination region (1385-1098); TRP1 ORF (2107-1433); CEN6/ARS4 (2797-2282); Amp resistance gene ORF (2931-3791); pUC origin (3936-4609).

[0023] Figure 6 shows the polylinker of plasmid pYD1 (SEQ ID NO: 7).

[0024] Figures 7a-i show a restriction map of plasmid pYD1.

DETAILED DESCRIPTION OF THE INVENTION

[0025] The description that follows makes citation to various references that may aid one of ordinary skill in the art to understand or practice various aspects of the present invention. Citation to such references is intended as a short-hand method to direct the skilled person to more detailed techniques and example materials and systems of the prior art that may be used to practice the present invention. Citation of a reference herein is not an admission that such a reference constitutes prior art for any determination of patentability.

[0026] As used herein, the singular forms "an," "a," and "the" used in the specification and claims include both singular and plural unless the content clearly dictates otherwise. In particular, those skilled in the art will recognize that while design and creation of catalytic materials and catalytic supports are described in terms of a single cell or other

biological support, more effective systems will include one or more cells or biological supports each expressing one or more receptor proteins.

[0027] As used herein, the term "anchoring protein" means a polypeptide sequence having an amino acid sequence domain, or combination of domains, that function(s) to engage the polypeptide with the surface of a biological particle, such as a cell, virus or virion shell. The polypeptide sequence need not, and typically does not, embody an entire protein as it would occur naturally in nature, but rather need only include a fragment or fragments of such proteins carrying a sufficient amount of the amino acid sequence as necessary to provide the anchoring domains. It is understood by one of skill in the art, that in certain cases, anchoring domains may include two or more contiguous sequences of amino acids that engage within a membrane of a cell separated by one or more contiguous sequences that do not engage with the membrane. In such cases, only the domains that engage with membrane and are necessary to provide the anchoring function need be included, while the amino acid sequences that do not engage with the membrane may be deleted altogether, or replaced by sequences that are heterologous to the naturally occurring protein.

[0028] One aspect of the invention provides a catalytic support material including a biological support that displays one or more receptor proteins on the biological support surface. This catalytic support provides uniform availability to catalysts bound to a ligand that will be tethered to a receptor protein. These catalysts may be organic or inorganic. Unlike prior art surface display catalysis technology, the catalysts are not limited to enzymes or proteins that can be fused to natural anchoring proteins and expressed by the biological support.

[0029] In one embodiment, the catalytic support material comprises a yeast cell and one or more receptor proteins displayed on the surface of the yeast cell, as represented by Figure 3. The receptor proteins are anchored to the surface of the cell by fusion to proteins that anchor to the cell wall by operation of native yeast cell mechanisms. For example, the agglutinin system and the flocculin system are well known to those skilled in the art and may be used in embodiments of the invention. Anchoring may be accomplished, for example, by creation of fusion proteins in which (1) the receptor proteins are fused to the C-terminal half of an α -agglutinin construct; (2) the receptor proteins are fused to the N-terminal half of an α -agglutinin construct; (3) the receptor proteins are fused to the C-terminal region of Flo1p; or (4) the receptor proteins are fused to the flocculation functional domain of Flo1p. Possible receptor proteins include avidin (SEQ ID NO: 26), a cellulose binding domain, a lectin, and antibody binding domains.

[0030] In one embodiment of the invention, the receptor proteins are introduced to the yeast cell by expression of one or more genes that have either been added to the yeast cell or added to a parent of the yeast cell. Genes may be added, for example, by transformation using lithium salts, spheroplasts, a gene gun, or electroporation.

[0031] Methods of making catalytic support materials are also provided herein. In one embodiment of the invention, a biological support expresses a receptor protein fused to a native yeast protein that is exported to the outer membrane of the yeast cell. One or more receptor proteins are displayed about the surface of the yeast cell. The receptor proteins may be identical proteins or they may be different proteins. The receptor proteins may be distributed uniformly about the surface of the yeast cell. The receptor proteins will bind

to ligands that may be attached to catalysts to be supported by the catalyst support systems.

[0032] In a further embodiment the invention includes a catalytic material. The catalytic material includes an organic or an inorganic catalyst bound to a ligand, which is in turn tethered to a receptor protein on a yeast catalytic support material as described herein. A catalytic material of the invention is shown in two-dimensional view in Figure 1. The catalyst forms a mono-layer on the yeast cell surface. This gives the properties of homogeneous catalysis, yet still allows efficient separation and ready recycling of the catalyst.

I. Cells and Other Biological Supports

[0033] A variety of biological cells are suitable for use in embodiments of the invention. Those skilled in the art will recognize that many strains of microorganisms are suitable for use in embodiments of the invention. Strains of yeast are preferred. Strains of the yeast *Saccharomyces cerevisiae* are particularly suitable in embodiments of the invention, in part because their genome has been extensively studied, and because they enjoy GRAS ("Generally Regarded As Safe") status with the Food and Drug Administration. *Saccharomyces cerevisiae* is recognized as a model eukaryote capable of rapid growth and with a versatile DNA transformation system.

[0034] Background information and exemplary methods and media for growing, testing and preserving yeast in general and *S. cerevisiae* in particular may be found, for example, in Sherman, F., "Getting Started with Yeast," Dept. of Biochem. and Biophysics, Univ. of Rochester Med. Sch. (August 2003) (adapted from Sherman, F., "Getting Started with Yeast," *Methods Enzymol.*, 350:3-41 (2002) (hereinafter "Sherman (I)"); and in Sherman,

F., "An Introduction to the Genetics and Molecular Biology of the Yeast *Saccharomyces cerevisiae*," Dept. of Biochem. and Biophysics, Univ. of Rochester Med. Sch. (1998) (modified from Sherman, F., "Yeast Genetics," *The Encyclopedia of Molecular Biology and Molecular Medicine*," 6:302-325 (edited by R.A. Meyers, Weinheim, Germany, 1997); and Burke, D., *et al.*, "Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual" (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2000); Colby, *et al.* "Engineering Antibody Affinity by Yeast Surface Display" *Methods Enzymol.* 388:348-58 (2004).

[0035] Some non-yeast microorganisms and virions also have display mechanisms that have been studied and that may be useful in embodiments of the invention. For example, *Escherichia coli* may be used for surface display of enzymes with the aid of a PgsA anchor. Narita, *et al.*, "Display of Active Enzymes on the Cell Surface of *Escherichia coli* using PgsA Anchor Protein and Their Application to Bioconversion," *Appl. Microbiol. Biotechnol.* (2005). Display of lipase using fadL as an anchor has been reported. Lee, S.H., *et al.*, "Display of Bacterial Lipase on the *Escherichia coli* Cell Surface by Using FadL as an Anchoring Motif and Use of the Enzyme in Enantioselective Biocatalysis" *Appl. & Env. Microbiol.* 70(9): 5074-80 (2004). Cell surface display of proteins on *Lactococcus lactis* has also been reported. Steidler, *et al.*, "Functional Display of a Heterologous Protein on the Surface of *Lactococcus lactis* by Means of the Cell Wall Anchor of *Staphylococcus aureus* Protein A" *Appl. & Env. Microbiol.* 64(1): 342-345 (1998).

[0036] In other embodiments, the biological support can be a virion. Virions may be eukaryotic viruses or bacteriophages, either as complete virion particles, or as so called

“naked” virions, which are viral particles devoid of the viral genome. Suitable eukaryotic viruses include those commonly used for genetic engineering purposes, including, for example, baculovirus, adenoviruses, adeno-associated viruses and retroviruses .

Similarly, suitable bacteriophages include, for example, λ phage (Lysogen), T4 phage, T7 phage, R17 phage, M13 phage, MS2 phage, G4 phage, P1 phage, P2 phage, N4 phage, ϕ 6 phage, and ϕ 29 phage. The filamentous phage of the M13, including but not limited to M13, fd, and f1, are preferred. Methods of displaying receptors on the surface of bacteriophages are well known in the art. Phage display is reported, for example, in Willats, W.G.T., "Phage Display: practicalities and prospects," *Plant Mol. Biol.* 50: 873-54 (2002); Bass, *et al.*, "Hormone Phage: An Enrichment Method for Variant Proteins with Altered Binding Properties," *Proteins: Structure, Function, and Genetics*, 8:309-314 (1990); and Zucconi, *et al.*, "Selection of Ligands by Panning of Domain Libraries Displayed on Phage Lambda Reveals New Potential Partners of Synaptojanin 1," *J. Mol. Biol.* 307: 1329-1339 (2001). Creation and use of avidin-displaying Baculovirus is reported by Rätty, *et al.*, "Enhanced Gene Delivery by Avidin-Displaying Baculovirus" *Mol. Therapy* 9(2):282-291 (2004). Surface display using bacteriophage T7 is reported in the "T7 Select® System Manual," TB178 Rev.B0203, copyright 2003 by Novagen, Inc.

II. Anchoring Proteins

[0037] Those skilled in the art will recognize that a variety of anchoring proteins may be used in embodiments of the invention. Ideally, anchoring proteins will not be essential to cellular functions or structures, thereby avoiding growth defects or functional issue with the cell or other biological support. Lee, *et al.*, "Microbial Cell-Surface Display" *Trends*

in *Biotech.* 21(1): 45-52 (2003). Lee, *et al.* reports beneficial characteristics of anchoring proteins:

[0038] A successful carrier should meet the following four requirements: it should have an efficient signal peptide or transporting signal to allow premature fusion protein to go through the inner membrane; it should have strain anchoring structure to keep fusion proteins on the cell surface without detachment; it should be compatible with the foreign sequences to be inserted or fused (i.e. the carrier should not become unstable on the insertion or fusion of heterologous sequences); and it should be resistant to attack by proteases present in the periplasmic space or medium.

[0039] Boder and Wittrup (*supra*) report two cell-surface receptors that may be fused to proteins of interest and anchored to the cell wall. These receptors, α -agglutinin and α -agglutinin, are normally used by the yeast to enable cell fusion prior to diploid formation.

[0040] Boder and Wittrup report using α -agglutinin in the development of yeast cell surface display of a functional anti fluorescein scFv and c-myc epitope tag on the cell wall of *S. cerevisiae*. They claim that this was accomplished by C-terminal fusion of the protein of interest to the Aga2 protein (SEQ ID NO: 8). The Aga2 protein is linked to the Aga1 protein subunit (SEQ ID NO: 2) by disulfide bonds. In turn, the Aga1 subunit is anchored to the cell wall by β -glucan covalent bonding. Boder and Wittrup further report construction of a vector in which the Aga2-scFv fusion is placed under the control of the inducible GAL1 promoter (SEQ ID NO: 9). A nucleic acid sequence encoding the Aga1 protein is given in SEQ ID NO: 10. A nucleic acid sequence encoding the Aga2 protein

is given in SEQ ID NO: 11. A nucleic acid sequence encoding an α -agglutinin gene (*sag1*) is reported in SEQ ID NO: 12.

[0041] A number of methods for anchoring these proteins have been described, as shown in Figure 1. These methods include glycosylphosphatidylinositol (GPI) anchor attachment of the C-terminal half of α -agglutinin, GPI anchor attachment of the N-terminal half of α -agglutinin (combined with sulfur bonding to the C-terminal portion), GPI anchoring of the C-terminal region of flocculin Flo1p, and N-terminal anchoring of flocculin Flo1p (without GPI anchoring). The GPI anchor length of the C-terminal region of flocculin Flo1p (SEQ ID NO: 3) may have a length, for example, of 42, 102, 146, 318, 428, and 1326 amino acids. Flo1p is encoded by, for example, the nucleic acid sequence of SEQ ID NO: 13. GPI anchors, which have a conserved core structure of protein-CO-NH-CH₂-CH₂-PO₄-6-mannose (Man) α 1,2-Man- α 1,6-Man- α 1,4-GlcN- α 1,6-inositol-PO₄-lipid, are discussed in Newman, *et al.*, "Gpi19, the *Saccharomyces cerevisiae* Homologue of Mammalian PIG-P, Is a Subunit of the Initial Enzyme for Glycosylphosphatidylinositol Anchor Biosynthesis," *Eukaryotic Cell*, 4(11): 1801-1807 (2005); Eisenhaber, *et al.*, "Enzymes and Auxiliary Factors for GPI Lipid Anchor Biosynthesis and Post-translational Transfer to Proteins," *BioEssays*, 25: 367-385 (2003); Kinoshita, T. & Inoue, N., "Dissecting and Manipulating the Pathway for Glycosylphosphatidylinositol-anchor Biosynthesis," *Curr. Opin. Chem. Biol.* 4: 632-638 (2000); McConville, M.J. & Menon, A.K., "Recent Developments in the Cell Biology and Biochemistry of Glycosylphosphatidylinositol Lipids," *Mol. Membr. Biol.* 17: 1-17 (2000); Orlean, P., "Biogenesis of Yeast Wall and Surface Components," p. 229-362, in Pringle, *et al.* (eds.), "Molecular and Cellular Biology of the Yeast *Saccharomyces*

cerevisiae" Cold Spring Harbor Press, Cold Spring Harbor, N.Y.; and Tiede, *et al.*, "Biosynthesis of Glycosylphosphatidylinositols in Mammals and Unicellular Microbes," *Biol. Chem.* 380:503-523. (1999).

[0042] Other anchoring proteins useful in yeast cells may be used in embodiments of the invention. These include, for example, but are not limited to, C-terminal end fragments of Cwp1p (SEQ ID NO: 14), encoded, for example, by the nucleic acid sequence of SEQ ID NO: 21; Cwp2p (SEQ ID NO: 15), encoded, for example, by the nucleic acid sequence of SEQ ID NO: 22; Tip1p (SEQ ID NO: 16), encoded, for example, by the nucleic acid sequence of SEQ ID NO: 23; Tir1p (SEQ ID NO: 17), encoded, for example, by the nucleic acid sequence of SEQ ID NO: 24; Sed1p (SEQ ID NO: 18), encoded, for example, by the nucleic acid sequence of SEQ ID NO: 38; YCR89-313 (SEQ ID NO:19), which is a fragment of YCR89 (SEQ ID NO: 25); YCR89-744 (SEQ ID NO:20), Flo1p-344, and Flo1p-596. Methods for use of these anchoring proteins and their efficacy are reported in van der Vaart, J. M., *et al.*, "Comparison of Cell Wall Proteins of *Saccharomyces cerevisiae* as anchors for Cell Surface Expression of Heterologous Proteins," *App. & Env. Microbiol.* 63(2): 615-620 (1997).

III. Receptor Proteins

[0043] A wide range of proteins have been reportedly displayed on cell surfaces. These proteins may be displayed outside the cell either by extension through the cell's plasma membrane or by covalent or noncovalent interactions with the cell membrane surface. Typically, yeast cells are armed by expressing a protein on the surface of the cell by fusing the protein with one of the anchoring proteins described above. Although it is not necessary, ideally a gene encoding the fused proteins may be incorporated into the

genome of the yeast cell. This incorporation may be accomplished by methods known to those skilled in the art. For example, the yeast cell may be transformed using lithium salt methods, integrative replicative vectors, spheroplasts, a "gene gun," or electroporation.

[0044] According to Kondo, supra, proteins that have reportedly been displayed on the surface of yeast cells using α -agglutinin bonding include *Rhizopus oryzae* glucoamylase, *Bacillus stearothermophilus* α -amylase, *Aspergillus aculeatus* β -glucosidase, *Trichoderma reesei* endoglucanase, *Rhizopus oryzae* lipase, and *Aequorea victoria* GFP (including BFP, ECFP, EYFP, Apoequorin, Hexa-His, ZZ, and Fab fragment of antibody). Proteins displayed using the α -Agglutinin system include single-chain antibodies and single-chain T-cell receptors. Proteins displayed using the C-terminus region of Flo1p include *Rhizopus oryzae* glucoamylase and EGFP. Proteins displayed using the flocculation functional domain of Flo1p include *Rhizopus oryzae* lipase and EGFP. Those skilled in the art will, with the benefit of this disclosure, recognize other proteins that may be displayed.

[0045] The identity of a suitable binding ligand will vary depending on the receptor protein exhibited by the catalytic support material. For example, if the catalyst support displays a lectin, an organic catalyst may be modified so that a glycan that bonds with that lectin is attached to a side chain on the catalyst.

[0046] The catalyst support may also display one member of an antigen antibody binding pair. The term "antibody" is used herein in the broadest scope to include any protein molecule having a variable region and a constant region where the variable region binds an antigen. Thus, the term antibody includes ScFv molecules of the IgG, IgM, and IgA class, single chain T-cell receptor molecules, as well as multi chain versions of the same

linked by disulfide bonds. When the catalyst support includes an anchoring protein fused to an antibody, the antibody is preferably a ScFv. , and in most such cases, the ligand will be the corresponding antigen. The antigen will include at least the epitope tag that binds the antibody, and may also contain one or more non epitope regions that can be used to tether the ligand or ligand catalyst pair. Kieke, *et al.* reports a number of polypeptide sequences that can be used as epitope tags. The proteins reported by Kieke can be fused to surface proteins and bound by antibodies. With the advantage of the disclosure herein, one skilled in the art would note that the antibodies of Kieke could be bound to catalysts and, in turn, tethered to a cell surface that displays those epitope tags.

[0047] Those skilled in the art will contemplate other receptor/ligand combinations that may be suitable for use in the invention. These include, for example avidin/biotin and streptavidin/biotin combinations.

[0048] A yeast cell expressing a receptor protein could serve as a catalytic support material for any catalyst that could be bound to the ligand designated to bind to the displayed receptor protein. A single yeast line could therefore be used to support, in turn, multiple catalysts.

Biotin/Avidin Interaction

[0049] In a preferred embodiment, biotin/avidin binding is used to tether the catalyst to the receptor protein. Biotin/streptavidin binding may also be used. These may be accomplished, for example, by displaying avidin or streptavidin on the surface of a yeast cell as the receptor protein and binding biotin to the catalyst. The cell and the catalyst are mixed, and the strong biotin/avidin or biotin/streptavidin binding interaction tethers the catalyst to the cell.

[0050] The biotin/avidin interaction has been extensively studied, as have the procedures and reagents needed for biotinylation. Biotin/avidin chemistry and biotin/streptavidin chemistry, including reagents and methods for biotinylation as well as detection of biotinylated molecules, is discussed in M. D. Savage, *et al.*, "Avidin-Biotin Chemistry: A Handbook" (Pierce Chemical Co., 1992). Creation of metalloenzymes using biotinylated metal catalysts incorporated with streptavidin is reported in Skander, *et al.*, "Chemical Optimization of Artificial Metalloenzymes Based on the Biotin-Avidin Technology: (S)-selective and Solvent-tolerant Hydrogenation Catalysts *via* the Introduction of Chiral Amino Acid Spacers" *Chem. Commun.*, 4815-4817 (2005).

[0051] Biotinylation of proteins (both antigens and antibodies) is used extensively in ELISA/EIA procedures. This extensive use provides a library of methods and reagents for those skilled in the art to determine how to best biotinylate a molecule. With the benefit of this disclosure, those skills may be used to biotinylate catalysts to be tethered to yeast cells. For example, Alpha Diagnostic International reports the following reagents and methods for biotinylation of a protein using long-arm biotin (Aminohexanoyl-biotin N-hydroxysuccinimide). The ADI biotinylation requires at least one free amino group on the biotinylated protein, and ideally the protein is free of amine-containing buffers (such as Tris) to improve coupling efficiency:

[0052] Reagents Provided in the ADI Kit:

1. 1 vial of Biotin (10 mg; Cat # 80301)
2. Conjugation Buffer, pH, 8.4, 100 ml, Cat # 80302
3. Stabilizing Buffer; 5 ml., Cat # 80303

Reagents Required But not Provided in the ADI Kit:

1. PBS, pH 7.4 for Dialyzing the conjugate (dissolve 0.26 g KH_2PO_4 , 2.17 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 8.71 g of NaCl in 1L H_2O).
2. Dialysis bag (Cut off size <10,000 kDa)
3. DMF for dissolving Biotin

Procedure

1. Dialyze the protein to be coupled in 1X conjugation buffer (0.1 M NaHCO_3 , pH 8.4) extensively. The protein concentration should be 1-10 mg/ml. If the protein is in pure form or in saline or in H_2O , it is possible to adjust the pH by adding 10X conjugation buffer and omitting dialysis.
2. Dissolve biotin in DMF at 10 mg/ml before use. Add dissolved biotin protein solution at a predetermined ratio to the protein solution slowly under continuous mixing. A ratio of 1:10 (biotin:protein) may be used for goat or rabbit antibodies. Mix it at room temp. for 1 h or overnight at 4°C.
3. Dialyzed the biotin-conjugate extensively against PBS at 4°C.
4. Add stabilizing buffer to the dialyzed conjugate (add 2 ml for each 500 ul of starting protein volume).
5. The conjugate can be kept at 4 °C for up to 6 months. Avoid freezing and thawing.

[0053] A typical nucleic acid sequence encoding avidin is provided in SEQ ID NO: 28.

A typical nucleic acid sequence encoding streptavidin is provided in SEQ ID NO: 29. A typical amino acid sequence of avidin is provided in SEQ ID NO: 26. A typical amino acid sequence of streptavidin is provided in SEQ ID NO: 27.

IV. Plasmids

[0054] A number of plasmids are suitable for expression of anchoring proteins, receptor proteins and fusions of the same. Many plasmids are also available for integration of the nucleic acids encoding these proteins into the yeast genome and for expression of the coding sequence. Expression plasmids typically contain a promoter element for expression of the nucleic acid encoding the protein. Expression plasmids may also contain signal peptides and transit peptide sequences for fusion to coding sequences so as to direct the expressed protein into the cellular transport machinery for export of the protein to the cell surface. Boder, E.T. & Wittrup, K.D., "Yeast Surface Display for Directed Evolution of Protein Expression, Affinity, and Stability," *Methods in Enzymology*, 25:430-444 (2000) (hereinafter "Boder & Wittrup (II)") reports construction of the pCT302 plasmid. The pCT302 plasmid (shown in Figure 4) allows expression, of a protein fusion to the Aga2p agglutinin mating protein. Expression is under the control of the GAL 1,10 galactose-inducible promoter. Both the 5' flanking sequence (SEQ ID NO: 4) and 3' flanking sequence (SEQ ID NO: 5) of the promoter have been reported. Boder & Wittrup (II) also reports methods for detection of protein anchored on the cell surface through use of the reported plasmid.

[0055] Another plasmid useful for yeast cell surface display is the pYD1 Yeast Display Vector Kit offered by Invitrogen. A map of pYD1 (SEQ ID NO: 6) is included as Figure 5. The polylinker of pYD1 (SEQ ID NO: 7) is included as Figure 6, and a restriction map is included as Figure 7. According to Invitrogen, "pYD1 is a 5.0 kb expression vector designed for expression, secretion, and display of proteins on the extracellular surface of *Saccharomyces cerevisiae* cells. Features of this vector allow regulated expression,

secretion, and detection of expressed proteins....". The vector contains the following elements:

- (a) *AGA2* gene from *Saccharomyces cerevisiae*. This gene encodes one of the subunits of the a-agglutinin receptor. Fusion of the gene of interest to *AGA2* allows secretion and display of the protein of interest.
- (b) *GAL1* promoter for regulated expression of the *AGA2* gene fusion.
- (c) Xpress™ epitope and V5 epitope for detection of the displayed protein.
- (d) Polyhistidine (6xHis) tag for detection and possible purification on metal chelating resin.
- (e) *TRP1* gene for selection in *Saccharomyces cerevisiae*.
- (f) *CEN6/ARS4* for stable, episomal replication in yeast.
- (g) Ampicillin resistance gene and the pUC origin for selection and replication in *E. coli*.

Methods of preparation and use of the pYD1 plasmid are presented in "pYD1 Yeast Display Vector Kit," Catalog No. V835-01, Version D, December 10, 2002, 25-0259, Invitrogen.

V. Catalysts

[0056] A variety of catalysts are useful in embodiments of the invention. To be suitable for use in the invention, catalysts should be able to be modified so that they are bound to a ligand that will be tethered (for example, either adsorbed or bound) to a receptor protein, which is in turn anchored to the cell surface. Ideally, catalysts should also not be harmful to or destructive of the yeast cell. Catalysts may be inorganic or organic.

[0057] For example, [$\{N,N'$ -Bis(3,5-di-tert-butylsalicylidene)1,2-cyclohexanediaminato(-2-)}cobalt(2)] can be modified so that a glycan is attached on a side chain of the catalyst. The appropriate lectin for this glycan is displayed on the yeast catalyst support. The glycan-bearing cobalt catalyst and the lectin-displaying yeast support are mixed, allowing the cobalt catalyst to adsorb to the yeast support. This immobilizes the cobalt catalyst on the yeast, allowing the cobalt catalyst to be used in oxidation reactions.

[0058] A number of useful catalysts for oxidation and reduction reactions may be bound to a ligand and tethered to a cell surface, particularly a yeast cell surface, as taught herein. For example, a catalyst may be a transition metal, a nitroxyl radical, or a supported noble metal. The following supported catalysts may be biotinylated according to methods known to those skilled in the art and set forth herein, then tethered to an avidin- or streptavidin-displaying yeast cell. The yeast cell would act as the support described in each case. Each catalyst is followed by a publication that describes that catalyst: chloromethyl polystyrene resin-1,10-phenanthroline ruthenium (Kang, Q., *et al.*, "Catalytic Oxidation of Alcohols with Polymer-supported Ruthenium Complex under Mild Conditions," *J. Organometallic Chem.*, 26:690 (2005)); poly(ethylene glycol)-supported nitroxyl radicals of the 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) family (Ferreira, P., *et al.*, "Catalytic Oxidation of Alcohols using Molecular Oxygen Mediated by Poly(ethyleneglycol)-supported Nitroxyl Radicals," *Applied Catalysis*, 61:3-4 (2005)) and Ciriminna, *et al.*, "Sol-gel Entrapped TEMPO for the Selective Oxidation of Methyl α -D-glucopyranoside," *Chem. Commun.*, 1441-1442 (2000); Rh₆- and Os₆- cluster catalysts (Pomogailo, S. I., *et al.*, "Synthesis and Catalytic Properties of Polymer-immobilized Noble Metal Clusters," *PMSE Preprints*, 93:945 (2005)); vanadium

catalysts (Kirihaara, M., *et al.*, "Aerobic Oxidation catalyzed by Polymer-supported Vanadium Compounds," *ITE Letters*, 5(5): 479-482 (2004); manganese porphyrin (Moghadam, M., *et al.*, "A Convenient Preparation of Polymer-supported Manganese Porphyrin and Its Use as Hydrocarbon Monooxygenation Catalyst," *J. Mol. Catalysis*, 217(1-2): 9-12 (2004); the Co(II) complex of bis(salicylideneethylenediamine) (CoSalen) (Finashina, E.D., *et al.*, "Oxidation of Catecholamines on Chitosan-immobilized Co(II) Salen Complexes," *Macromolecular Symposia*, 204: 205-217 (2003); copper (ii) ions (Owsid, I., *et al.* "Immobilized Cu(II) Ions as the Oxidation Catalysts," *Zeszyty Naukowe Politechniki Slaskiej, Chemia*, 146:113-116 (2001); homochiral palladium complexes polymeric 2',6- and 6'-anchored 2-diphenylphosphino-1-1'-binaphthyl (MOP) ligands (Hocke, H. & Uozumi, Y., "PS-PEG Resin-supported Palladium-MOP Complexes. Application in Asymmetric π -allylic Reduction," *Tetrahedron*, 60(41): 9297-9306 (2004); oxazaborolidine (Franot, C., *et al.*, "A Polymer-bound Oxazaborolidine Catalyst: Enantioselective Borane Reductions of Ketones," *Tetrahedron: Asymmetry*, 6(11):2755-66 (1995).

[0059] Multiple catalysts may be tethered to a single cell surface. If the cell bears only a single type of receptor protein, then this may be accomplished by binding each catalyst to identical ligands. For example, if the cell surface displays avidin, then two or more distinct catalysts may be attached to separate biotin molecules. A similar result may be accomplished by expressing more than one kind of receptor protein on the surface of a cell, then providing different catalysts that are bound to different ligands; each ligand may be designed to bind to a different receptor protein.

Examples

[0060] The following examples are intended to guide those skilled in the art in the practice of this invention.

Example 1

[0061] Example 1 provides amino sulfonamide ruthenium complex oxidation of sec-phenethyl alcohol. The catalyst biotin complex is prepared by a method reported in J. Collot, *et al.*, *J. Am. Chem Soc.* 125 (2003). This complex is mixed with an avidin displaying yeast in water to prepare the immobilized inorganic catalyst/yeast complex, and the yeast is separated from any unbound catalyst by centrifugation. The oxidations are carried out under the following conditions: nitrogen atmosphere at room temperature for 90 hours: 62.5 millimoles of phenethyl alcohol, and 75 millimoles of *tert*-butylhydroperoxide are mixed in a mixture of 500 ml of water and 100 ml of acetone. To this mixture is added the immobilized complex which has 0.25 millimoles of the biotin metalloenzyme complex. On completion of the reaction oxidized phenethyl alcohol is observed. The yeast metalloenzyme complex is removed from the reaction mixture by filtration or centrifugation.

Example 2

[0062] Example 2 provides biotinylated catalyst synthesis. Biotin (0.1 mol) is dissolved in 90 mL of *N,N*-dimethylformamide at 60 °C. (One synthesis of the amide is reported in S. Amslinger, *et al.*, *Tetrahedron* 60 (2004) 11565-11569.) The solution is then cooled to room temperature and 0.1 mol of *N,N'*-carbonyldiimidazole is added under nitrogen (approximately 7 hours). Carbon dioxide evolution ceases when the reaction is complete. An equimolar amount (0.1 mol) of the given NH₂-R, where R is an appropriate catalytic

site, is added in 125mL of DMF and stirred for 24 hours. For example, the NH₂-R compound could be amino-TEMPO (2,2,6,6-Tetramethyl-1-oxy-4-piperidiny)amine. DMF is then removed under vacuum at 60 °C and the product can then be purified by flash chromatography, column chromatography or recrystallization.

Example 3

[0063] Example 3 reports construction of an avidin yeast cell surface display plasmid. Truncated avidin DNA (SEQ ID NO: 41), encoding truncated avidin protein (SEQ ID NO: 42) is made by 30 cycles of PCR amplification using the following primers: Forward, CGAACTGGATCCTCTCCCAGAAAGTGCTCGCTG (SEQ ID NO: 30) and reverse, CGGATCCTCGAGTCACTCCTTCTGTGTGCG (SEQ ID NO: 31) using a chicken avidin cDNA clone as a template for PCR. The amplified DNA is cut with BamHI and XhoI and ligated into the vector pYD1 (Invitrogen) cut with BamHI and XhoI.

[0064] The resulting clones are transformed into *E. coli* and transformants are isolated and sequenced to verify the proper in frame fusion of avidin and the aga2 protein. A correct construct is then transformed into *Saccharomyces cerevisiae* EBY100 (Invitrogen) using the Invitrogen *S.c.* EasyComp Kit for preparing competent *S. cerevisiae* cells. The expression of avidin-Aga2 fusion protein is accomplished by growing transformed cells in YNB-CAA medium containing 2% galactose for at least about 4 hours according to the Invitrogen instructions. Cell surface display of the avidin molecule is verified using biotin conjugated with a fluorescence molecule such as Atto 425-biotin, available from Fluka.

Example 4

[0065] Example 4 provides TEMPO catalyzed oxidation of glucopyranoside. A tempo/biotin complex is prepared by the method of Example 2. This complex is then mixed with a suspension of yeast cells displaying the avidin protein to form the immobilized Tempo complex; uncomplexed Tempo is separated from the yeast by centrifugation. The complex is mixed with a aqueous solution of alpha methyl glucoside (25mM) and NaOCl (50mM) at pH 10 and 2 °C. On completion of the reaction, alpha methyl glucuronic acid is observed. The catalyst complex is easily separated from the mixture by centrifugation. Further information on the TEMPO catalyst may be obtained, for example, from R. Ciriminna, *et al.*, "Sol-gel entrapped TEMPO for the selective oxidation of methyl- α -D-glucopyranoside," *J. Chem. Soc., Chem. Comm.*, 1441 - 1442 (2000).

Example 5

[0066] Example 5 presents coupled enzyme reactions. Biotinylated forms of glucose oxidase and catalase are prepared as discussed above. These are mixed in a ratio of 6 IU of catalase to 1 IU of glucose oxidase. Yeast cells displaying avidin are added to this mixture in an amount that absorbs all of the enzymatic activities. The catalytic complex is added to a 10% dextrose solution with the pH controlled at 6.0 at about 25 °C. Aeration is applied for about 24 hours. Upon completion of this reaction gluconic acid is observed and all of the hydrogen peroxide produced during the reaction will have been degraded by the catalase. The catalyst complexes are easily recovered by filtration.

[0067] Further guidance on enzyme behavior and catalyst creation in general may be obtained from M. T. Reetz, "Controlling the Enantioselectivity of Enzymes by Directed

Evolution: Practical and Theoretical Ramifications," *Proc. Natl. Acad. Sci. U. S. A.*, 101: 5716-5722 (2004), Wilson, M. E. and Whitesides, G. M., "Conversion of a Protein to a Homogeneous Asymmetric Hydrogenation Catalyst by Site-Specific Modification with a Diphosphinerhodium(I) Moiety", *J. Am. Chem. Soc.*, 100: 306-307 (1978); and C. M. Thomas, *et al.*, "Aqueous Oxidation of Alcohols Catalyzed by Artificial Metalloenzymes based on the Biotin-Avidin Technology," *J. Organomet. Chem.*, 690: 4488 (2005).

Example 6

[0068] Example 6 presents construction of a T7 bacteriophage with streptavidin displayed on its surface. Truncated streptavidin DNA (SEQ ID NO: 43), encoding truncated streptavidin protein (SEQ ID NO: 44), is made by 30 cycles of PCR amplification using the following primers: forward, GCGAATTCAGACCCCTCCAAGGACTCG (SEQ ID NO: 32) and reverse, GCAAGCTTCTACTGCTGAACGGCGTC (SEQ ID NO: 33) and using *Streptomyces avidinii* (ATCC 27419D) genomic DNA as a template.

[0069] The amplified DNA is cut with EcoRI and HindIII and ligated into Novagen's T7Select 10-3b EcoRI/HindIII vector arms supplied in the T7Select Cloning Kit from Novagen. The ligation reactions are added to the T7 packaging extracts for in vitro packaging according to the procedure supplied by Novagen. Various clones are selected for sequencing and ones that had the proper fusion of the streptavidin gene to the T7 capsid protein 10B are selected for further work. Correct T7 phage with avidin displayed on their surface were amplified and phage particles recovered.

Example 7

[0070] Example 7 reports construction of a plasmid for display of streptavidin on the cell surface of a strain of *E. coli*. A truncated *fadL* gene (SEQ ID NO: 40) is cloned by PCR amplification using the following primers: forward, GGAATTCATGGTCATGAGCCAGAAAACC (SEQ ID NO: 34) and reverse, GCTCTAGAACGATTCTGTGCAGGAAC (SEQ ID NO: 35), and using *E. coli* genomic DNA (ATCC 700926D).

[0071] This PCR product is cut with *EcoRI* and *XbaI* and cloned into pTrc99A cut with *EcoRI* and *XbaI* to make a plasmid called pTrcFadL. The streptavidin DNA is made by PCR using the following primers: forward, GCTCTAGAGACCCCTCCAAGGACTCG (SEQ ID NO: 36), and reverse, GCAAGCTTCTACTGCTGAACGGCGTC (SEQ ID NO: 33) and using *Streptomyces avidinii* (ATCC 27419D) genomic DNA as a template.

[0072] The streptavidin DNA is cut with *XbaI* and *HindIII* and ligated into pTrcFadL digested with *XbaI* and *HindIII*. This produces a plasmid called pTrcFadL-streptavidin (SEQ ID NO: 37) which is an inframe fusion of the *fadL* gene product (SEQ ID NO: 40) and the streptavidin protein. The plasmid is transformed into TOP10 *E. coli* competent cells (Invitrogen) and selected on LB with ampicillin (100 mg/l). Individual colonies are tested to determine if the expression of the streptavidin on the cell surface is achieved by growing the transformed cells in LB with ampicillin (50mg/l) and isopropyl β -D-1 thiogalactopyranoside (IPTG).

[0073] The cell surface display of the streptavidin molecule is verified using biotin conjugated with a fluorescence molecule such as Atto 425-biotin (available from Fluka). Correct *E. coli* with streptavidin displayed on the cell surface is grown in the presence of

ampicillin (50 mg/l) and various concentrations of IPTG to make cells with streptavidin displayed on their cell surface.

[0074] The start codon of the truncated fadL gene used herein begins at base 8 of SEQ ID NO: 39. Avidin and streptavidin used in this and other examples are truncated due to the removal of peptide leader sequences. One skilled in the art would recognize, based on the PCR primer sequences, that these proteins would be produced in truncated form.

[0075] The scope of the claims should not be limited by the preferred embodiments and examples, but should be given the broadest interpretation consistent with the description as a whole". Those patents and publications discussed herein should be viewed as indicative of the level of skill in the art, though no admission is made that any document is a prior art reference.

We claim:

1. A catalytic material composition comprising:
 - a biological particle being a virion or cell having a surface;
 - an anchoring protein that includes an anchoring domain engaged with the surface;
 - a receptor protein fused to the anchoring protein and having a ligand binding domain disposed from the surface;
 - a ligand bound to the ligand binding domain; and
 - a non-biological catalyst bound to the ligand.
2. The composition of claim 1 wherein a plurality of different receptor proteins are bound to a plurality of anchoring proteins.
3. The composition of claim 1 wherein said biological particle is selected from the group consisting of a yeast cell, a bacterial cell and a bacterial virion.
4. The composition of claim 3 wherein said virion is a bacteriophage selected from the group consisting of: λ phage, T4 phage, T7 phage, R17 phage, M13 phage, MS2 phage, G4 phage, P1 phage, P2 phage, N4 phage, ϕ 6 phage, and ϕ 29 phage.
5. The composition of claim 3 wherein said biological particle is a microorganism of a genus selected from the group consisting of *Escherichia*, *Corynebacteria*, *Brevibacteria*, *Bacillus*, *Saccharomyces*, *Candida*, *Pichia*, and *Lactococcus*.

6. The composition of claim 5 wherein said microorganism is a yeast cell of the strain *Saccharomyces cerevisiae*.

7. The composition of claim 1 wherein said biological particle is a yeast cell and said anchoring protein and receptor protein comprise a yeast cell surface display system.

8. The composition of claim 7 wherein said yeast cell surface display system comprises polypeptide components selected from the group consisting of a glycosylphosphatidylinositol (GPI) anchor fused to α -agglutinin; a GPI anchor fused to an N-terminal region of α -agglutinin combined with disulfide bonding of a C-terminal region of α -agglutinin on the cell surface; a GPI-anchor fused to a C-terminal region of Flo1p; and a N-terminal anchoring of the flocculation functional domain of Flo1p without GPI.

9. The composition of claim 1 wherein said receptor protein comprises a protein selected from the group consisting of avidin and streptavidin; and said ligand is biotin.

10. The composition of claim 1 wherein said receptor protein comprises a lectin and said ligand is a glycan that binds to said lectin.

11. The composition of claim 1 wherein said catalyst is selected from the group consisting of $[\{N,N'\text{-Bis}(3,5\text{-di-tert-butylsalicylidene})1,2\text{-cyclohexanediaminato}(-2-)\}\text{cobalt}(2)]$, chloromethyl polystyrene resin-1,10-phenanthroline ruthenium, poly(ethylene glycol)-supported nitroxyl radicals of the 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) family, Rh6- and Os6- cluster catalysts, vanadium catalysts, manganese porphyrin, the Co(II)

complex of bis(salicylideneethylenediamine), nitroxyl radicals, transition metals, copper (ii) ions, homochiral palladium complexes, noble metals, polymeric 2',6- and 6'-anchored 2-diphenylphosphino-1-1'-binaphthyl (MOP) ligands, and oxazaborolidine.

12. A catalyst support composition comprising:

- a biological particle being a virion or cell having a surface;
- an anchoring protein that includes an anchoring domain engaged with the surface;
- a receptor protein fused to the anchoring protein and having a ligand binding domain disposed from the surface; and
- a ligand bound to the ligand binding domain, said ligand having a domain for binding to a selected catalyst.

13. A method for catalyzing a chemical reaction comprising contacting a reactant for the reaction with a catalytic material composition comprising a biological particle being a virion or cell having a surface, an anchoring protein that includes an anchoring domain engaged with the surface, a receptor protein fused to the anchoring protein and having a ligand binding domain disposed from the surface, a ligand bound to the ligand binding domain, and a non-biological catalyst bound to the ligand.

14. A method for making a catalyst support composition comprising displaying a non-biological catalyst from the surface of a biological particle being a virion or cell.

15. The method of claim **14** wherein displaying the non-biological catalyst from the surface of the biological particle comprises:

displaying at least one receptor protein on the surface of the biological particle by
 providing a membrane spanning fusion protein comprising an interior anchoring
 protein fused to exterior displayed receptor protein;
 providing at least one non-biological catalyst molecule bound to a ligand; and
 binding said ligand to said receptor protein to form the catalytic material
 composition.

16. The method of claim **14** wherein said biological particle is selected from the group consisting of a yeast cell, a bacterial cell and a bacterial virion.

17. The method of claim **16** wherein said virion is a bacteriophage selected from the group consisting of λ phage, T4 phage, T7 phage, R17 phage, M13 phage, MS2 phage, G4 phage, P1 phage, P2 phage, N4 phage, ϕ 6 phage, and ϕ 29 phage.

18. The method of claim **16** wherein said biological particle is a microorganism of a genus selected from the group consisting of *Escherichia*, *Corynebacteria*, *Brevibacteria*, *Bacillus*, *Saccharomyces*, *Candida*, *Pichia*, and *Lactococcus*.

19. The method of claim **14** wherein said biological particle comprises a receptor protein fused to an anchoring protein that is part of a yeast cell-display system.

20. The method of claim **19** wherein said yeast cell surface display system is selected from the group consisting of a glycosylphosphatidylinositol (GPI) anchor fused to α -agglutinin; a GPI anchor fused to a N-terminal region of α -agglutinin combined with disulfide

bonding of a C-terminal region of α -agglutinin on the cell surface; a GPI-anchor fused to a C-terminal region of Flo1p; and a N-terminal anchoring of the flocculation functional domain of Flo1p without GPI.

21. The method of claim **19** wherein said yeast display system comprises a recombinant nucleic acid sequence that encodes said anchoring protein fused to said receptor protein operably configured with a promoter sequence to express the anchoring protein fused to the receptor protein in the yeast cell and with a transport sequences encoding a peptide domain operable to transport the anchoring protein fused the receptor protein to the surface of the yeast cell.

22. The method of claim **21** wherein the recombinant nucleic acid is integrated into a chromosome of the cell.

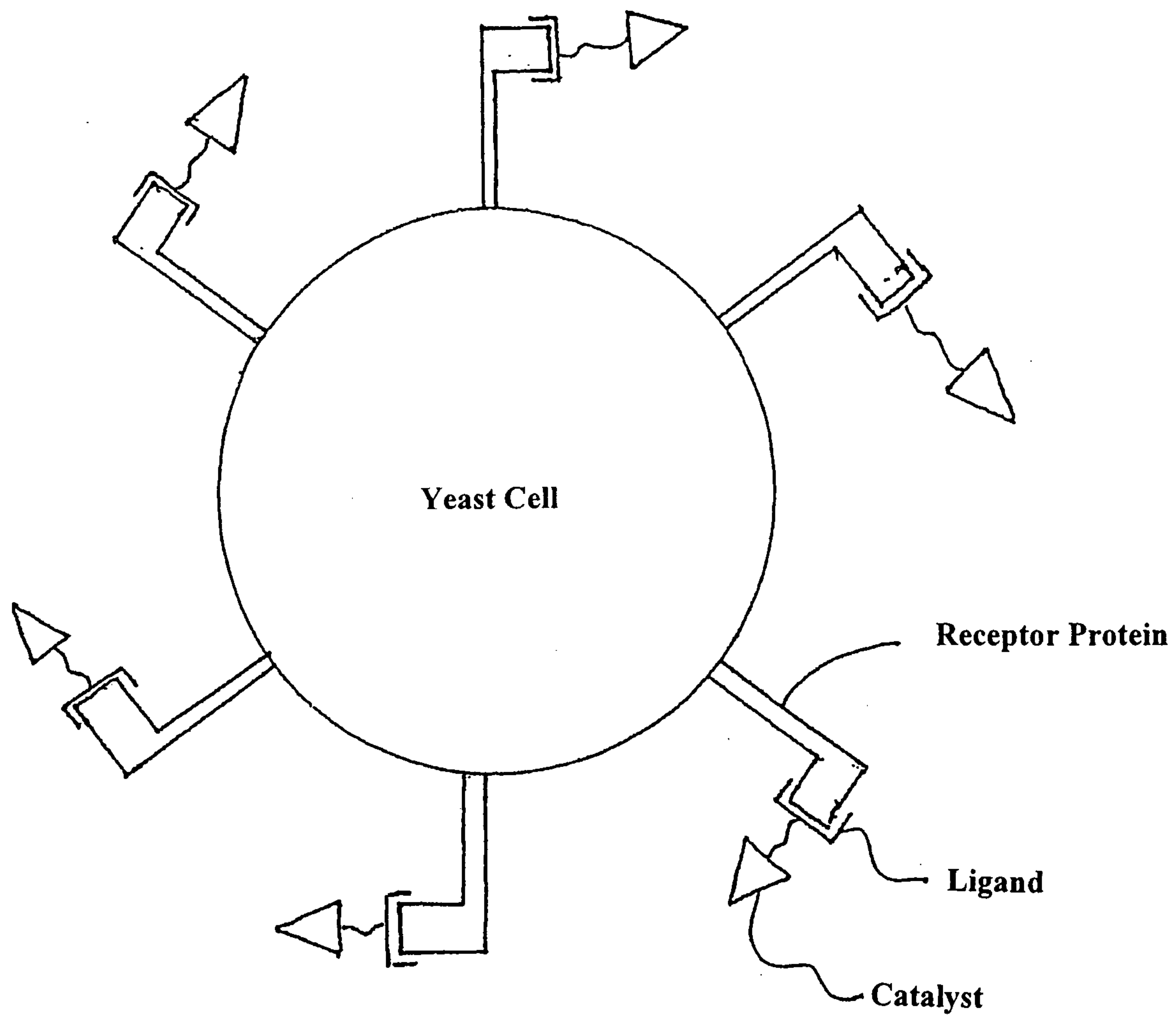
23. The method of claim **15** wherein said receptor protein is selected from the group consisting of streptavidin and avidin; and said ligand is biotin.

24. The method of claim **15** wherein said receptor protein is lectin and said ligand is a glycan that binds with said lectin.

25. The method of claim **15** wherein said catalyst molecules are selected from the group consisting of [{N,N'-Bis(3,5-di-tert-butylsalicylidene)1,2-cyclohexanediaminato(-2-)}cobalt(2)], chloromethyl polystyrene resin-1,10-phenanthroline ruthenium, poly(ethylene glycol)-supported nitroxyl radicals of the 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)

family, Rh₆- and Os₆- cluster catalysts, vanadium catalysts, manganese porphyrin, the Co(II) complex of bis(salicylideneethylenediamine), nitroxyl radicals, transition metals, copper (ii) ions, homochiral palladium complexes, noble metals, polymeric 2',6'- and 6'-anchored 2-diphenylphosphino-1-1'-binaphthyl (MOP) ligands, and oxazaborolidine.

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**Figure 1******Not drawn to scale**

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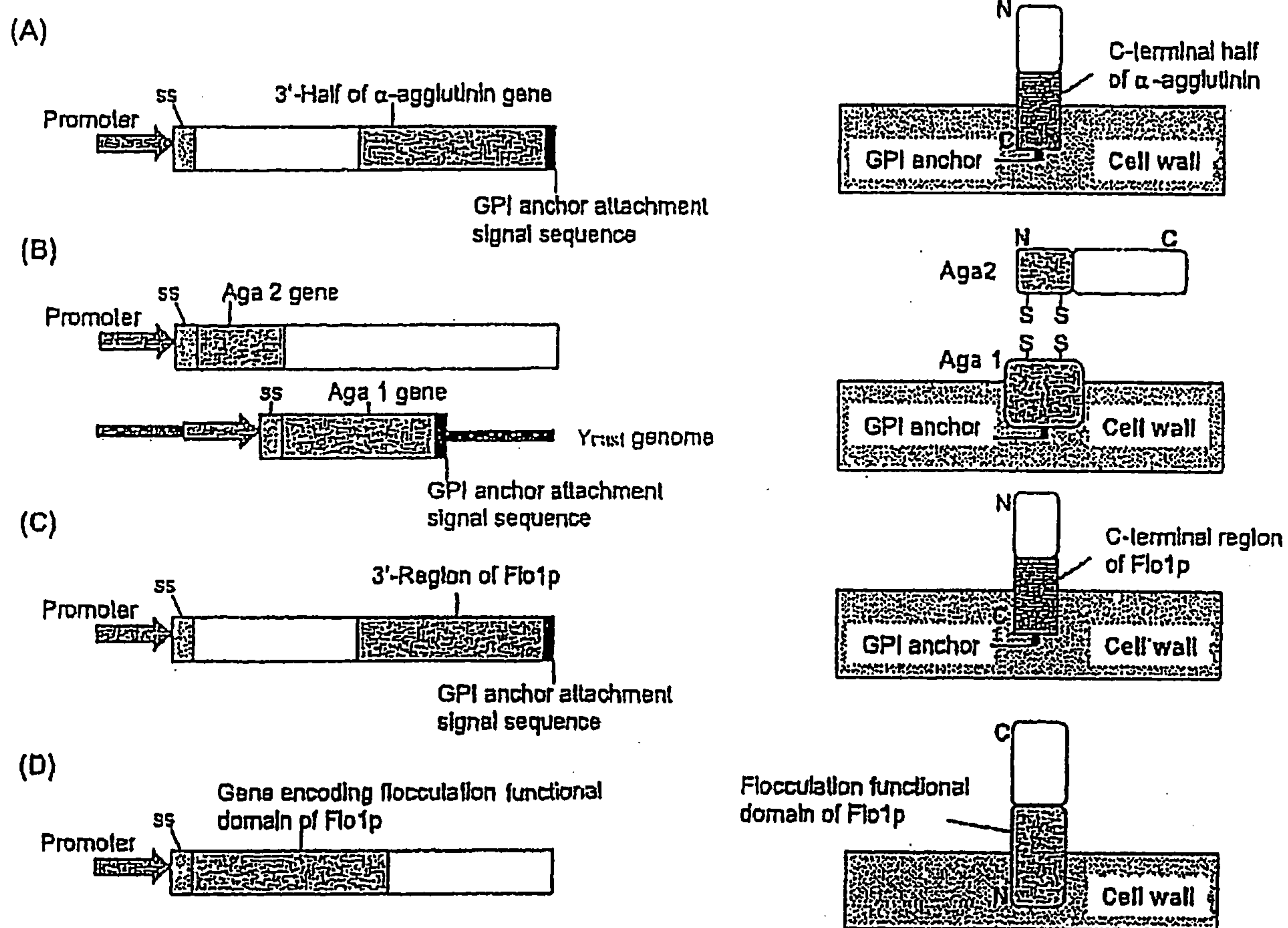


Figure 2

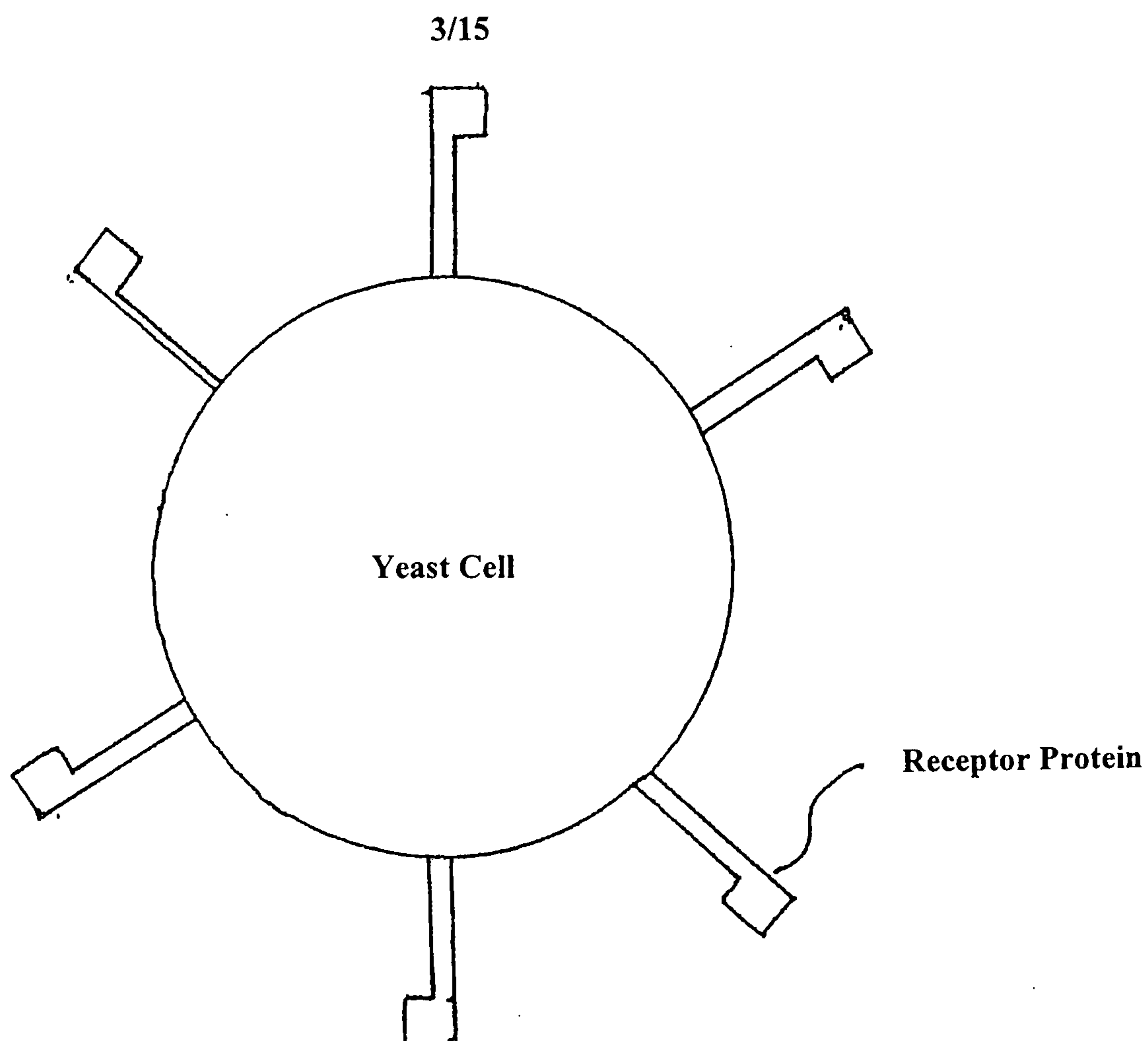
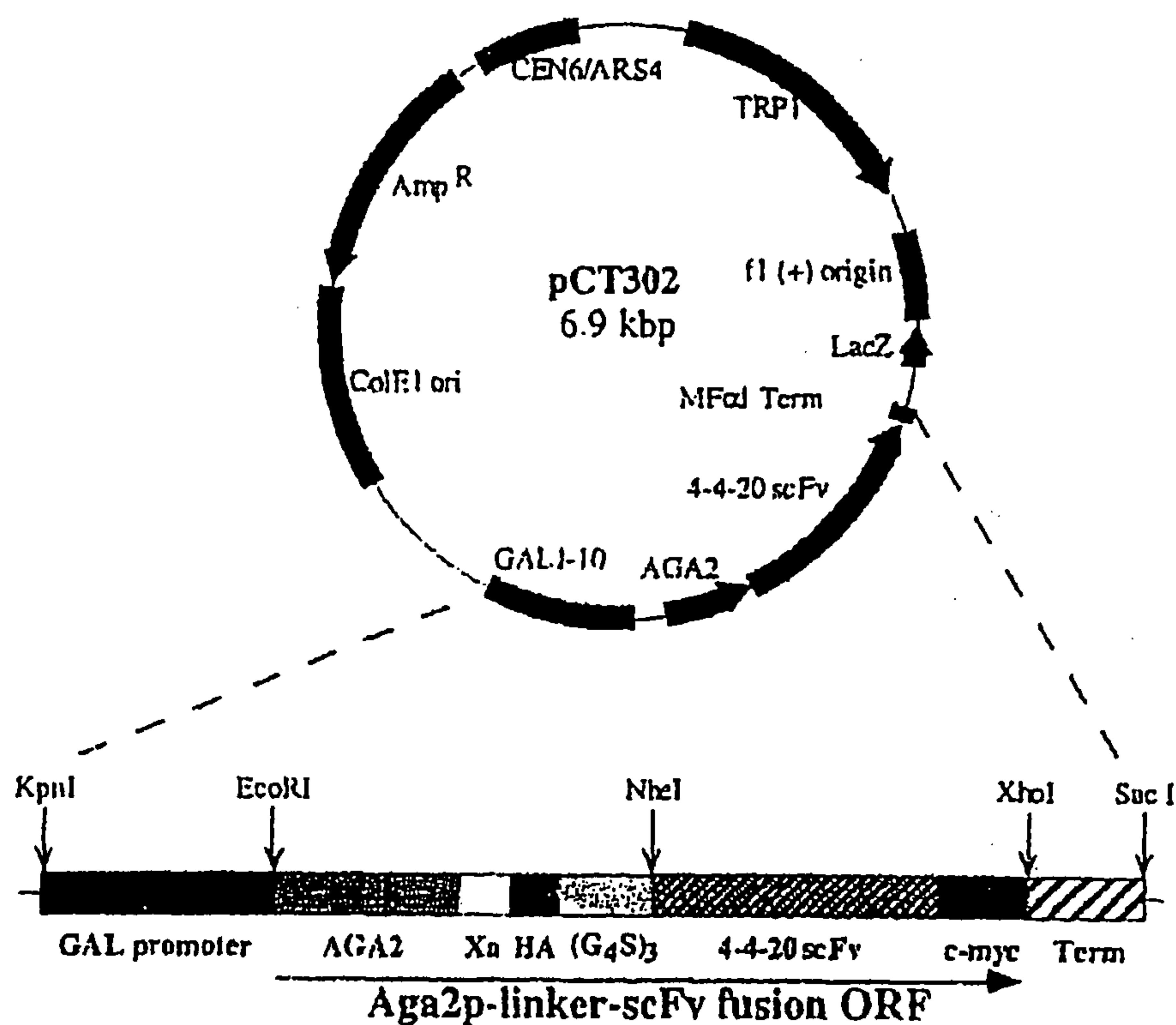


Figure 3*

***Not drawn to scale**

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**N-terminal flanking sequence:**

ATAAACACACAGTATGTTTAAAGGACAATAGCTCGACGATTGAAGGTAGATACCCATAC
 I N T Q Y V F K D N S S T I E G R Y P Y -
 Aga2p <-|-> Linker <-|Factor Xa^|-> HA

PstI/
 GACGTTCCAGACTACGCTCTGCAGGCTAGTGGTGGTGGTGGTTCGGTGGTGGTGGTCT
 D V P D Y A L Q A S G G G G S G G G G S -
 epitope tag <-|-> Linker

NheI/ AatII/
 GGIGGTGGTGGTTCGCTAGCGACGTCGTTATGACTCAAACACCACTATCACTTCCTGTT
 G G G G S A S D V V M T Q T P L S L P V -
 <-|-> VL of 4-4-20 scFv (SEQ ID NO:4)

C-terminal flanking sequence:

XhoI/ /BglII
 TCCTCAGAACAAAAGCTTATTTCTGAAGAAGACTTGTAATAGCTCGAGATC
 S S E Q K L I S E E D L *(SEQ ID NO:5)-
 <-| |> c-myc epitope tag <-|

Figure 4

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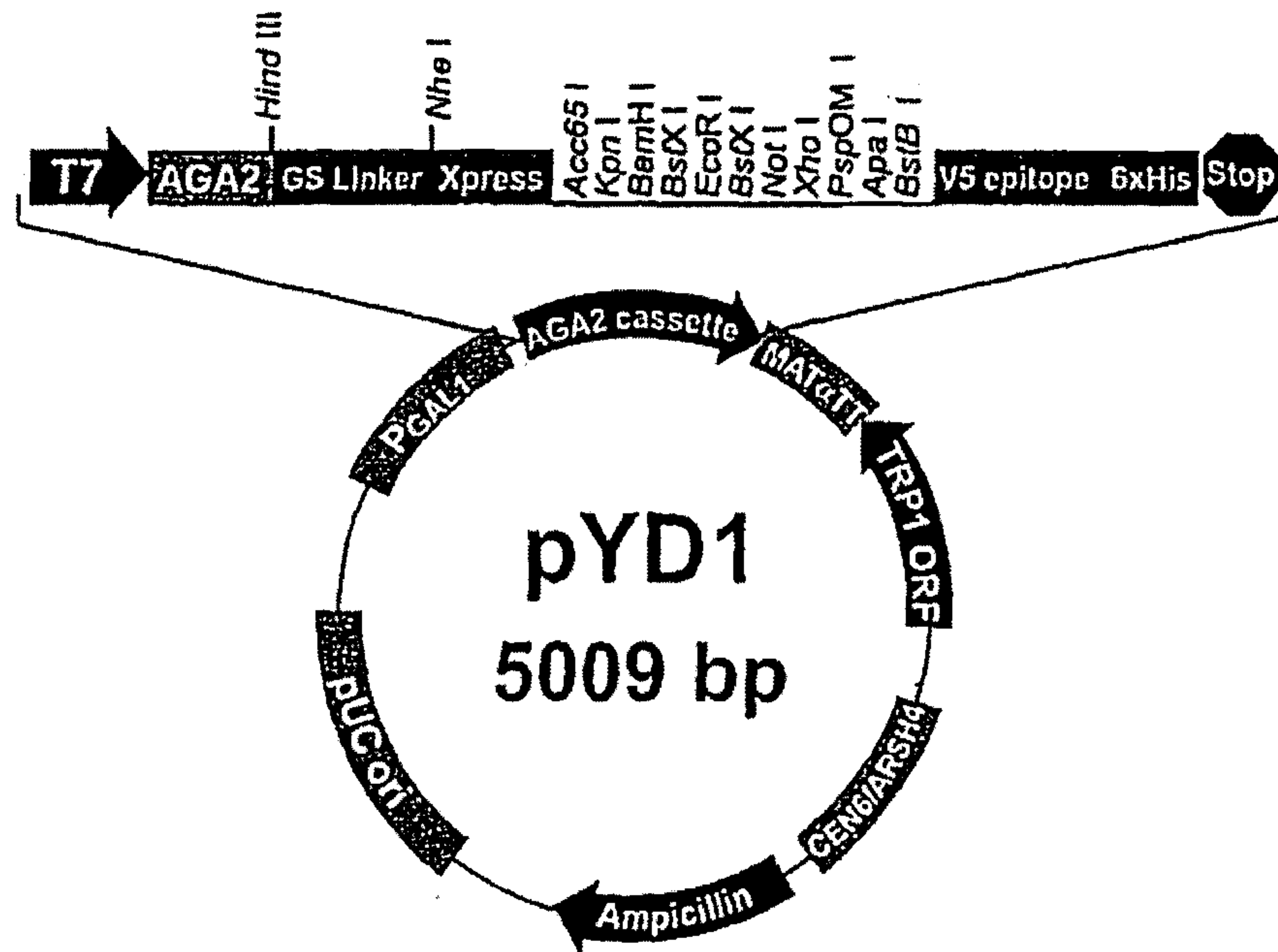


Figure 5

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transcriptional start

320 A A A C T G C A T A A C C A C T T T A A C T A A T A C T T T C A A C A T T T T C G G T T T G T A T T A C T T C T T A T T C A A A T G T A A T A A A A G T A T C

400 A A C A A A A A A T T G T T A A T A T A C C T C T A C T T T A A C G T C A A G G A G A A A A A C C C C G G A T C G G A C T A C T A G C A G C T G T A A T A

T7 promoter/priming site

480 C G A C T C A C T A T A G G G A A T A T T A A G C T A A T T C T A C T T C A T A C A T T T T C A A T T A A G A T G C A G T T A C T T C G C T G T T T T

Aga2 signal peptide

Met Gln Leu Leu Arg Cys Phe

Start of Aga2 mature peptide

565 T C A A T A T T T C T G T T A T T G C T T C A G T T T A G C A C A G G A A C T G A C A A C T A T A T G C G A G C A A A T C C C C

Ser Ile Phe Ser Val Ile Ala Ser Val Leu Ala Gln Glu Leu Thr Thr Ile Cys Glu Gln Ile Pro

Cleavage site

621 T C A C C A A C T T T A G A A T C G A C G C C G T A C T C T T T G T C A A C G A C T A C T A T T T T G G C C A A C G G G A A G G C A

Ser Pro Thr Leu Glu Ser Thr Pro Tyr Ser Leu Ser Thr Thr Thr Ile Leu Ala Asn Gly Lys Ala

pYD1 Forward priming site

687 A T G C A A G G A G T T T T T G A A T A T T A C A A A T C A G T A A C T T T G T C A G T A A T T G C G G T T C T C A C C C C T C A

Met Gln Gly Val Phe Glu Tyr Tyr Lys Ser Val Thr Phe Val Ser Asn Cys Gly Ser His Pro Ser

End of Aga2 mature peptide

Hind III

Pst I

751 A C A A C T A G C A A A G G C A G C C C A T A A A C A C A C A G T A T G T T T A A G C T T C T G C A G G C T A G T G G T G G T

Thr Thr Ser Lys Gly Ser Pro Ile Asn Thr Gln Tyr Val Phe Lys Leu Leu Gln Ala Ser Gly Gly

Gly-Ser Linker

Nhe I

819 G G T G G T T C T G G T G G T G G T T C T G G T G G T G G T T C T G C T A G C A T G A C T G G T G G A C A G C A A A T G

Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ala Ser Met Thr Gly Gly Gln Gln Met

Xpress™ epitope

Acc65 I Kpn I BamH I

BstX I EcoR I

Pst I

885 G G T C G G A T C T G T A C G A C G A T G A C G A T A A G G T A C C A G A T C C A G T G T G G A A T T C T G C A G A T A T

Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Val Pro Gly Ser Ser Val Val Glu Phe Cys Arg Tyr

Enterokinase recognition site

Enterokinase cleavage site

BstX I Not I Xho I Xba I PspOM I Ape I BstB I

V5 epitope

951 C C A G C A C A G T G G C G G C C G C T C G A G T C T A G A G G C C C T T C G A A G G T A A G C C T A T C C C T A A C C C T C T C

Pro Ala Gln Trp Arg Pro Leu Glu Ser Arg Gly Pro Phe Glu Gly Lys Pro Ile Pro Asn Pro Leu

Polyhistidine region

Pme I

1017 C T C G G T C T C G A T T C T A C G C G T A C C G G T C A T C A T C A C C A T C A C C A T T G A G T T A A A C C G C T G A

Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His His His ***

pYD1 Reverse priming site

1080 T C T G A T A C A A C A G T G T A G A T G T A A C A A A A T C G A C T T T G T T C C A C T G T A C T T T A G C T C G T A C A A A A T A C A A T A C T T

1160 T T C A T T T C T C G T A A A C A A C A T G T T T T C C C A T G T A A T A T C C T T T C T A T T T T C G T T C C G T T A C C A A C T T T A C A C A T A C T

(SEQ ID NO: 7)

*The two Pst I sites are unique to the multiple cloning site

Figure 6

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Restriction Map

Enzyme	# of cuts	Positions
Acc65I	1	915
AccI	1	2140
Accl	35	14 21(c) 108 737(c) 963(c) 967 1074 1504 2035(c) 2056 2828(c) 2875 2974(c) 3083(c) 3160(c) 3204 3325(c) 3371 3562(c) 3653(c) 4015 4024(c) 4159 4269(c) 4390(c) 4409(c) 4564(c) 4655 4676 4683 4726(c) 4750 4760 4816(c) 4919(c)
AcsI	8	937 1320 1456 1940 2206 2344 2456 2756
Acyl	3	640 1562 3179
AflIII	3	1033 1179 4607
AgeI	2	72 1039
AluI	22	145 472 504 798 1137 1248 1347 1576 1593 1711 2116 3366 3429 3529 4050 4307 4443 4669 4787 4946 4968 5001
Alw44I	3	1393 3047 4293
AlwI	14	463 899 917(c) 930 2294 2651(c) 3080 3084(c) 3401 3864(c) 3865 3961(c) 3963 4049
AlwNI	2	594 4198
AosI	1	3496
Apal	1	986
ApaLI	3	1393 3047 4293
ApoI	8	937 1320 1456 1940 2206 2344 2456 2756
AseI	6	271 2095 2608 3544 4779 4838
AsnI	6	271 2095 2608 3544 4779 4838
Asp700	2	2598 3119
Asp718	1	915
AspEI	1	3719

Figure 7a

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AspHI	7	1397 2028 2048 2153 3051 3136 4297
AsuII	1	989
AvaI	2	970 1596
Avall	4	2074 2796 3355 3577
Avill	1	3496
BamHI	1	922
BanI	3	915 3766 4863
BanII	2	986 1604
BbrPI	2	2148 2785
BbsI	4	49 59(c) 2222 2567(c)
BbvI	14	481 779 1280(c) 1698(c) 2103(c) 3285(c) 3676 3979(c) 4185(c) 4188(c) 4278 4697 4715 4796
BcgI	4	526(c) 868(c) 1843 3181(c)
Bfal	12	142 466 758 809 860 977 2021 2664 3526 3861 4114 5006
BglI	1	3601
BmyI	10	986 1397 1604 1987 2028 2048 2153 3051 3136 4297
BpmI	1	3650
BpuAI	4	49 59(c) 2222 2567(c)
BsaAI	3	2138 2148 2785
BsaHI	3	640 1562 3179
BsaI	2	1027 3653
BsaJI	6	451 1017 1771 2799 4447 4868
BsaWI	5	72 1039 3423 4254 4401
BsgI	2	1697(c) 1830
BsiEI	7	77 967 2204 3201 3350 4273 4697
BsiHKA I	7	1397 2028 2048 2153 3051 3136 4297
BsiYI	10	20 38 457 991 1018 4129 4408 4574 4592 4766
BsII	10	20 38 457 991 1018 4129 4408 4574 4592 4766
BsmAI	4	1027 1497 2877(c) 3653
BsmFI	1	1642(c)
Bsp120I	1	982

Figure 7b

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Bsp1286I	10	986 1397 1604 1987 2028 2048 2153 3051 3136 4297
BspHI	3	2260 2879 3887
BspMI	1	1671
BspWI	13	324 767 1599 2032 2052 2627 3601 3989 4561 4675 4742 4826 4870
BsrBI	5	21 969(c) 2877(c) 4678(c) 4919
BsrDI	3	692 3485 3659(c)
BsrFI	3	72 1039 3634
BsrI	13	873 926(c) 1620(c) 1767 3074 3244(c) 3513 3556 3674 4080 4192(c) 4205(c) 4813
Bst1107I	1	2141
BstBI	1	989
BstNI	7	182 920 2801 4448 4461 4582 4870
BstUI	11	79 105 1035 2310 2828 3160 3653 3983 4564 4762 4764
BstXI	2	933 959
BstYI	8	891 922 3072 3089 3857 3869 3955 3966
Bsu36I	1	1863
CfoI	16	107 1543 2312 2828 3160 3497 3590 3983 4092 4266 4366 4433 4703 4736 4764 4829
Cfr10I	3	72 1039 3634
Csp45I	1	989
Csp6I	9	645 897 916 1037 1128 1141 2473 3237 5009
DdeI	13	1274 1376 1422 1440 1537 1717 1863 2278 2615 3218 3758 3924 4333
DpnI	20	296 457 893 924 1080 2288 2658 3038 3074 3091 3349 3395 3413 3754 3859 3871 3949 3957 3968 4043
DpnII	20	294 455 891 922

Figure 7c

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		1078 2286 2656 3036 3072 3089 3347 3393 3411 3752 3857 3869 3947 3955 3966 4041
DraI	6	1069 2452 2761 3141 3833 3852
DraII	4	982 983 2225 2796
DrdI	1	4505
DsaV	12	180 451 918 1957 2799 3181 3532 4228 4446 4459 4580 4868
EaeI	4	671 964 3326 4768
EagI	1	964
Eam1105I	1	3719
EarI	5	154(c) 2407(c) 2620(c) 2920(c) 4724(c)
EclXI	1	964
Eco57I	3	560(c) 3053 4065(c)
EcoO109I	4	982 983 2225 2796
EcoRI	1	937
EcoRII	7	180 918 2799 4446 4459 4580 4868
EcoRV	2	949 1823
Fnu4HI	26	14 108 470 768 964 967 1294 1712 2036 2056 2117 2975 3204 3299 3326 3665 3993 4199 4202 4267 4410 4565 4683 4686 4704 4785
FnuDII	11	79 105 1035 2310 2828 3160 3653 3983 4564 4762 4764
FokI	5	241 1842(c) 3280 3567 3748
FspI	1	3496
HaeII	2	4367 4737
HaeIII	16	185 673 966 984 2038 2181 2227 3328 3595 3675 4133 4536 4567 4585 4596 4770
HgaI	6	47(c) 648 1551(c) 3187 3917(c) 4495(c)
HgiAI	7	1397 2028 2048 2153 3051 3136 4297
HhaI	16	107 1543 2312 2828

Figure 7d

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		3160 3497 3590 3983 4092 4266 4366 4433 4703 4736 4764 4829
HinP1I	16	105 1541 2310 2826 3158 3495 3588 3981 4090 4264 4364 4431 4701 4734 4762 4827
HincII	2	656 3177
HindII	2	656 3177
HindIII	1	796
HinfI	19	45 130 208 482 634 973 1027 1366 1371 1474 1672 1749 1798 2017 2667 4237 4633 4708 4773
HpaII	14	73 453 1040 1958 3182 3424 3534 3601 3635 4039 4229 4255 4402 4891
HphI	15	36 62(c) 614(c) 736(c) 1042(c) 1048(c) 1538(c) 1643(c) 2058(c) 2152(c) 2992(c) 3027 3233(c) 3649 3876
ItaI	26	14 108 470 768 964 967 1294 1712 2036 2056 2117 2975 3204 3299 3326 3665 3993 4199 4202 4267 4410 4565 4683 4686 4704 4785
KpnI	1	919
Ksp632I	5	154(c) 2407(c) 2620(c) 2920(c) 4724(c)
MaeI	12	142 466 758 809 860 977 2021 2664 3526 3861 4114 5006
Maell	8	434 721 2137 2147 2784 3117 3490 3906
MaellI	18	24 176 539 717 1101 1219 2158 2298 2416 3058 3246 3399 3457 3788 4071 4187 4250 4846
Mbol	20	294 455 891 922 1078 2286 2656

Figure 7e

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		3036 3072 3089 3347 3393 3411 3752 3857 3869 3947 3955 3966 4041
MbolI	15	54 59(c) 171 2227 2424 2439 2567(c) 2637 2671(c) 2937 3046 3124 3879 3950(c) 4741
McrI	7	77 967 2204 3201 3350 4273 4697
MfeI	1	1964
MluI	1	1033
MluNI	1	673
MnII	32	43 61 72 111 155(c) 431 630 759 973(c) 1022 1027 1511 1690(c) 1766(c) 1869 2049 2180 2184 2193 2238 2323 3346(c) 3552(c) 3699 3780 4180 4430(c) 4504 4713(c) 4763 4861 4991
MscI	1	673
MseI	37	216 271 301 338 413 431 500 530 794 1068 1285 1408 1532 1756 2095 2131 2254 2324 2451 2511 2531 2608 2707 2733 2751 2760 3140 3505 3544 3779 3832 3846 3851 3903 4779 4838 4976
MslI	4	1806 2948 3307 3466
MspA1I	6	472 1076 3083 4024 4269 4787
MspI	14	73 453 1040 1958 3182 3424 3534 3601 3635 4039 4229 4255 4402 4891
MunI	1	1964
MvaI	7	182 920 2801 4448 4461 4582 4870
MvnI	11	79 105 1035 2310 2828 3160 3653 3983 4564 4762 4764
MwoI	13	324 767 1599 2032

Figure 7f

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		2052 2627 3601 3989 4561 4675 4742 4826 4870
NciI	5	453 1959 3183 3534 4230
NdeII	20	294 455 891 922 1078 2286 2656 3036 3072 3089 3347 3393 3411 3752 3857 3869 3947 3955 3966 4041
NheI	1	859
NlaIII	12	867 1183 1193 2264 2883 3276 3312 3390 3400 3891 4611 4957
NlaIV	14	186 917 924 984 2798 2832 3422 3633 3674 3768 4535 4579 4865 4895
NotI	1	964
NspI	2	1183 4611
NspV	1	989
PaeR7I	1	970
PinAI	2	72 1039
PleI	9	39(c) 476(c) 981 1482 1757 1792(c) 2675 4231(c) 4716
PmaCI	2	2148 2785
PmeI	1	1069
PmlI	2	2148 2785
PpuMI	1	2796
Psp1406I	3	721 3117 3490
PstI	2	806 946
PvuI	1	3350
PvuII	2	472 4787
RcaI	3	2260 2879 3887
RsaI	9	1 646 898 917 1038 1129 1142 2474 3238
SapI	2	2620(c) 4724(c)
Sau3AI	20	294 455 891 922 1078 2286 2656 3036 3072 3089 3347 3393 3411 3752 3857 3869 3947 3955 3966 4041
Sau96I	12	184 982 983 2074 2180 2225 2796 3355 3577 3594 3673 4534

Figure 7g

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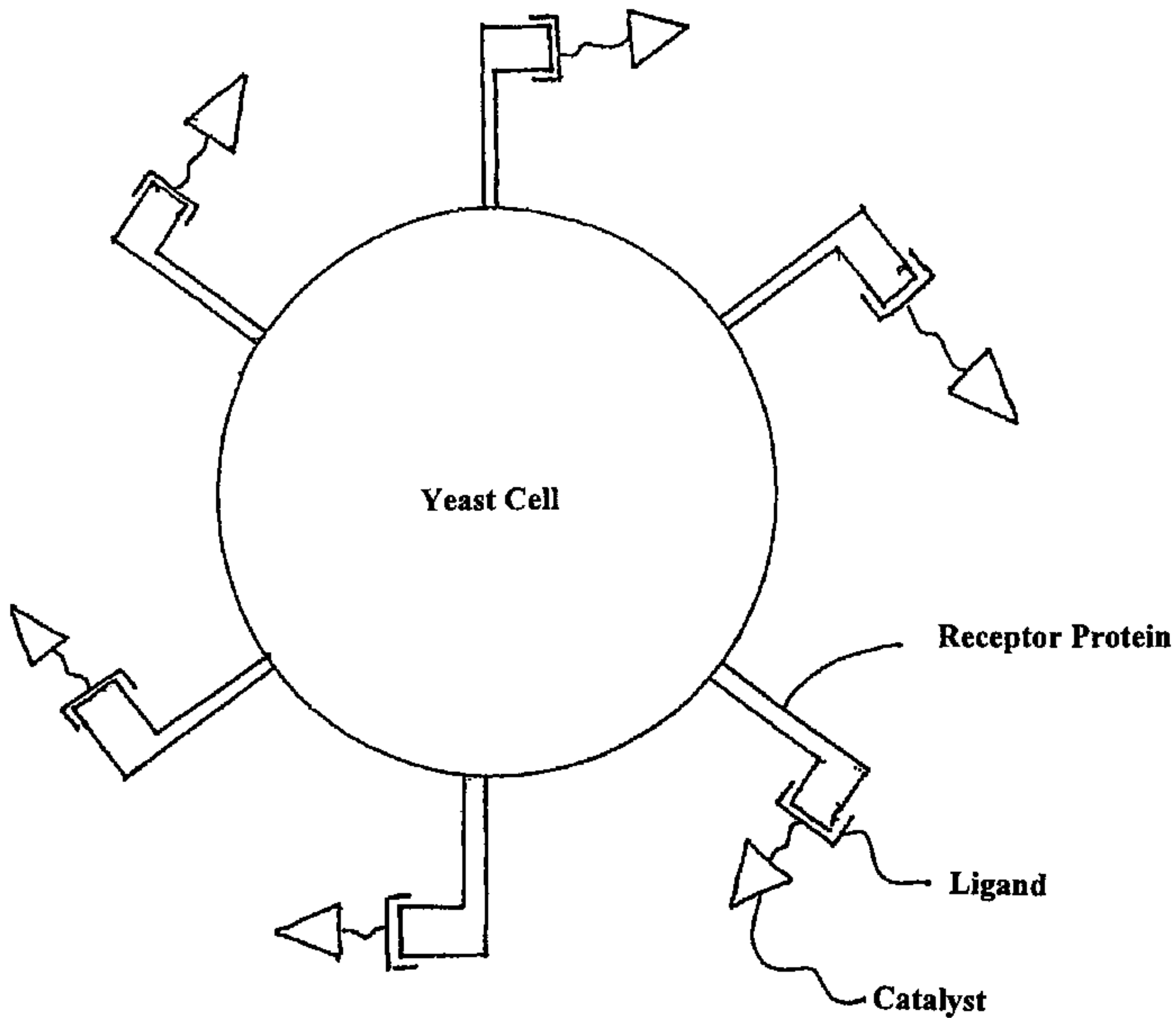
Scal	1	3238
ScrFI	12	182 453 920 1959 2801 3183 3534 4230 4448 4461 4582 4870
SfaNI	7	524(c) 1552 2020 3018(c) 3267 3458(c) 4510(c)
Sfcl	6	488 802 942 3473 4151 4342
Sful	1	989
Snol	3	1393 3047 4293
Sspl	4	498 560 706 2914
Swal	1	2761
TaqI	11	637 971 989 1025 1111 1369 1631 2200 2527 3065 4509
TfiI	10	130 208 634 1027 1366 1371 1672 2017 4633 4773
Thal	11	79 105 1035 2310 2828 3160 3653 3983 4564 4762 4764
Tru9I	37	216 271 301 338 413 431 500 530 794 1068 1285 1408 1532 1756 2095 2131 2254 2324 2451 2511 2531 2608 2707 2733 2751 2760 3140 3505 3544 3779 3832 3846 3851 3903 4779 4838 4976
Tsp509I	35	168 213 268 407 506 527 732 937 1280 1286 1309 1320 1329 1382 1456 1940 1964 2092 2206 2336 2344 2456 2598 2708 2734 2756 2762 2768 3286 3541 3847 4835 4910 4927 4973
XbaI	2	976 2020
XhoI	1	970
XhoII	8	891 922 3072 3089 3857 3869 3955 3966
XmaIII	1	964
XmnI	2	2598 3119

Figure 7h

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No cuts: AatI, AatII, AccII, AflII, AscI, Aspl, AvrII, BclI, BfrI, BglII, BlnI, BpuI102I, BsaBI, BseAI, BsiWI, BsmI, BspDI, BspEI, BsrGI, BssHII, BstEII, CclII, ClaI, DraII, DsaI, Ecl136II, Eco47III, EcoNI, Esp3I, EspI, HpaI, Kasi, KspI, Mami, MroI, NaeI, NarI, NcoI, NdeI, NgoMI, NruI, NsiI, PacI, PflMI, PpuI0I, RsrII, SacI, SacII, Sall, SexAI, SfiI, SgrAI, SmaI, SnaBI, SpeI, SphI, SspBI, StuI, StyI, Tth111I, Van9II, XcmI, XmaI

Figure7i



***Not drawn to scale**